Exhibit B

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Human Antibodies from Transgenic Mice

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We have used homologous recombination in ES cells to engineer B cell-deficient mice that are incapable of expressing endogenous immunoglobulin heavy and kappa light chain genes. We find that B cell development in these mutant mice can be rescued by the introduction of human germline-configuration heavy- and kappa light-chain minilocus transgenes. The transgenes rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation in response to antigen stimulation; thus recapitulating both stages of the humoral immune response using human, rather than mouse, sequences. The mice can be immunized; and human sequence, antigen specific, monoclonal antibodies can be obtained using conventional rodent hybridoma technology. These animals are also of interest for studying the normal processes of immunoglobulin gene expression. We discuss the example of heavy chain class switching, which has not been previously observed within an autonomous transgene.

KEYWORDS: *immunoglobulin genes, human antibodies, class switching, somatic mutation, targeted mutation, transgenic mice*

INTRODUCTION

The defining events in B cell differentiation are the assembly of immunoglobulin (Ig) heavy and light chain variable (V) region genes from multiple germline gene segments, and the resulting cell surface deposition of an Ig receptor capable of transducing antigenic signals. Early, antigen-independent B cell development takes place in the bone marrow or fetal liver, and is characterized by D to J_H, followed by V_H to DJ_H rearrangements of the heavy chain, and V_L to J_L joining of the light chain genes. The large number of potential V(D)J combinations, together with imprecision in joining of the recombination breakpoints and addition of template-independent nucleotides (N sequences) to the junctions, produces an extensive repertoire of antibody specificities. This results in the production of a heterogeneous population of newly formed B cells, each of which expresses a unique Ig receptor reactivity on its cell surface. Mature, surface Ig^+ B cells migrate from the bone marrow to the peripheral lymphoid organs where further differentiation is driven by encounter with antigen in conjunction with antigen specific T cells. This T cell dependent maturation results in further diversification of the antibody repertoire through the process of somatic mutation, leading to selection of high affinity mutations, as well as modification of the antibody's effector function as a result of class switching.

The central role of Ig heavy chain protein in promoting early B cell differentiation has been well documented in mice bearing gene targeted mutations [1–6]. Inability to express the heavy chain [2–6], or display it on the cell surface [1], results in an early block to B cell differentiation and the concomitant absence of mature, surface Ig^+, B cells from the bone marrow and periphery. Such mutations are of value not only for the information to be derived from the mutant phenotypes themselves, but also in their capacity to provide a null

background for expressing Ig transgenes. The Ig transgenes reported in the literature to date have been miniloci rather than intact copies of a complete locus. These minilocus transgenes represent either discreet subsets of a natural Ig locus, or more complicated constructs assembled by piecing together multiple relevant functional units from one or more individual loci. Despite the complexity of the natural Ig loci—exemplified by the large number of different types of transcriptional, recombinational, and coding elements contained within them—we and others have shown that subsets of the intact immunoglobulin loci can undergo V(D)J joining [7–15], and that these relatively short sequences can, to some extent, functionally complement mutations of the endogenous mouse loci by rescuing B cell development [16–20].

In this review we discuss our development of a minilocus transgenic mouse that expresses a functional repertoire of human sequence antibodies in the absence of endogenous mouse heavy-, and κ light-chain expression. This technology is being applied toward the development of *in vivo* therapeutics. In addition, the fact that these minilocus transgenes are able to carry out many of the normal functions of the intact immunoglobulin loci helps to define the location and extent of sequences important for those functions. We discuss the example of cis-acting sequences involved in heavy chain class switching.

IMMUNOGLOBULIN HEAVY CHAIN AND KAPPA LIGHT CHAIN-DEFICIENT MICE

B Cell-Deficient Mice

We designed the Ig heavy chain targeting construct (Fig. 1) to delete all four J_H gene segments, as well as the adjacent D_{Q52} gene segment ("J_HD" mutation). Mice homozygous for J_H deletion completely lack surface Ig expressing (sIg^+) B cells in the bone marrow and periphery [4] (Fig. 2 A,B), and are devoid of serum IgM or IgG. The B cell progenitors ($B220^+IgM^-$) remaining in the bone marrow of J_HD mutants are large $CD43^+$ cells (Fig. 2 C,D); consistent with an early block to B cell differentiation at the D_H to J_H rearrangement stage [21]. This is virtually identical to the B lineage phenotype of mice deficient in the recombination activating genes (RAG-1 and 2), in which B cell differentiation is also blocked at the D_H to J_H rearrangement stage by an inability to initiate VDJ rearrangement [2, 3].

Interestingly, κ light chain rearrangements, which typically follow heavy chain assembly, are detectable in these [4] and other heavy chain deficient mice [22], demonstrating that heavy chain rearrangement or expression is not required for κ light chain assembly to take place. This is analogous to the observation that TCRα light chains, which are expressed subsequent to TCRβ during development, can rearrange in the thymocytes of TCRβ-deffcient mice [3] . The observed order of Ig rearrangement (in which assembly of the heavy chain typically precedes that of light chains) is apparently the result of a stochastic, rather than a stepwise process. Thus, light chain genes preferentially rearrange during, or following, the μ dependent differentiation of large $CD43^+$ to small $CD43^-$ pre-B cells; however, there is no direct coupling to μ rearrangement or expression, and κ VJ joining does occur in less mature cell types.

Ig Kappa Light Chain Deficient Mice

Light chains comprise two isotypes, κ and λ, encoded by separate gene families. Rearrangement of the κ locus typically precedes that of λ, and individual B cell progenitors

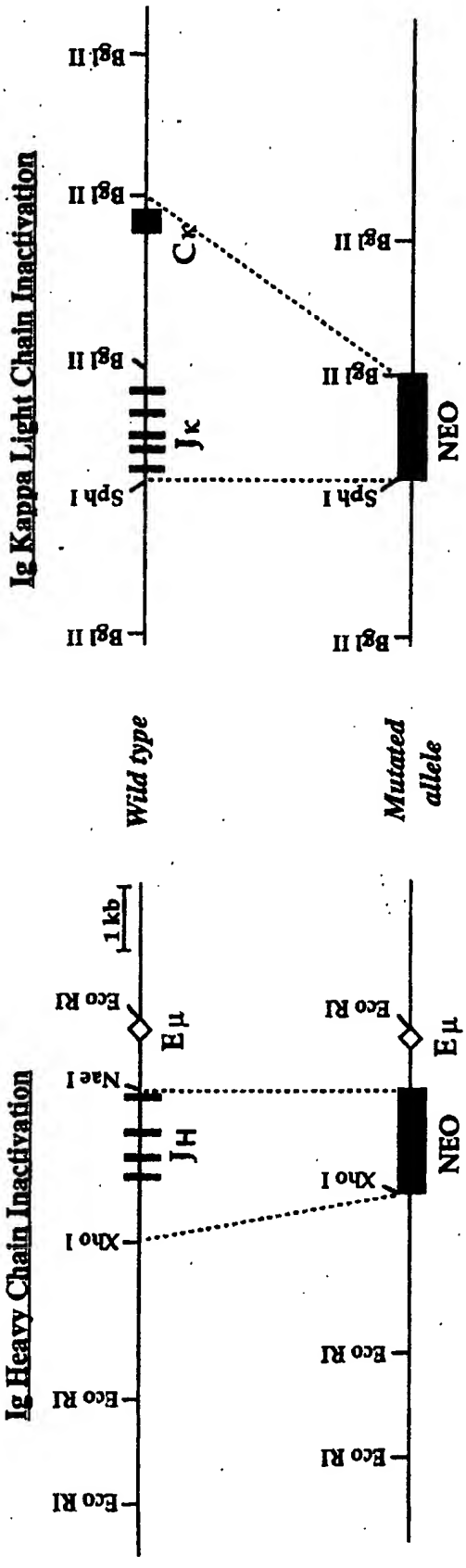


FIGURE 1 Targeted inactivation of endogenous mouse Ig loci. Schematic diagrams are shown of portions of the wild type murine heavy and kappa light chain loci, and of each mutated locus following homologous recombination in ES cells with targeting constructs. Dashed lines indicate those genomic sequences which have been deleted and replaced by the selectable marker *neo*. Closed boxes represent exons and the open diamond is the Ig heavy chain intron enhancer.

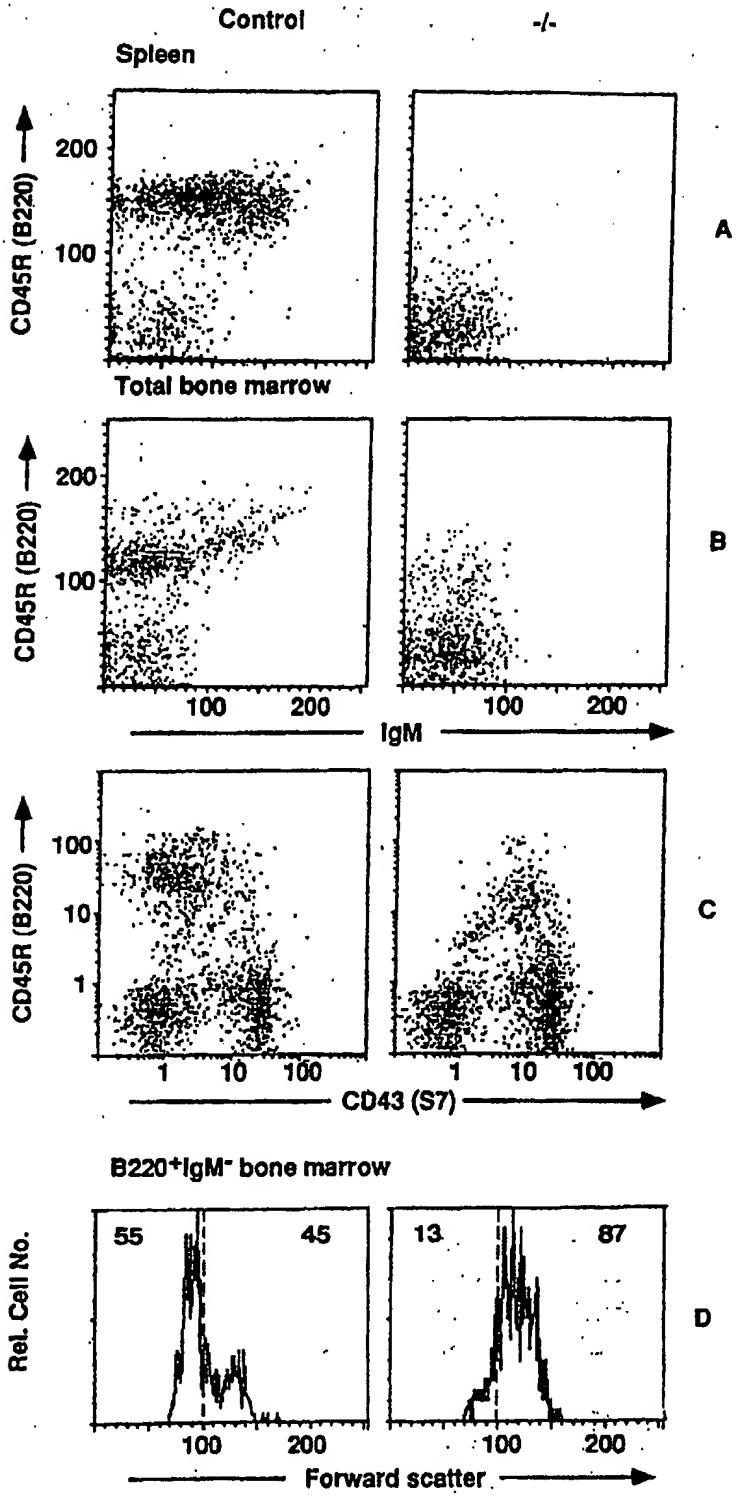


FIGURE 2 Flow cytometric analysis of cells from spleen and bone marrow of homozygous J_HD mutants (−/−) and control littermates (heterozygous mutants and wild type littermates had identical staining profiles). Four mice were analyzed from each genotype. Spleen cells (A) were stained with fluorescein-anti CD45R and APC-anti-IgM. Bone marrow cells (B, C, and D) were stained with fluorescein-anti-CD43, PE-anti-CD45R, and biotin-anti-IgM. Biotin conjugates were revealed by Streptavidin Cy-Chrome; dead cells were excluded by PI staining. In the representative dot plots shown, each dot indicates an individual cell.

productively rearrange and express only one of the two isotypes [23] to generate B cells which are either κ^+ or λ^+ (isotype exclusion) [24]. To reduce the expression of endogenous Ig proteins, we have specifically inactivated the κ locus and left the λ locus intact. However, this single mutation has a profound effect on overall light chain expression because of differential contribution of the two different loci to the light chain repertoire. In wild-type mice, typically ~95% of B cells express κ light chains while only ~5% express λ. This imbalance in light chain usage mirrors a corresponding imbalance in the extent of V region diversity of the two loci: in contrast to the over 150 functional V regions for the κ locus [25], there are only three V_λ gene segments [26].

We inactivated the κ locus by deletion of Jκ, Cκ, and intervening sequences in ES cells [27] ("JCκD" mutation; Fig. 1). Mice heterozygous for the deletion contain approximately twice as many λ^+ B cells as wild type mice (Fig. 3B). Homozygous mutants contain exclusively λ^+ B cells, and completely lack κ light chains (Fig. 3 A,B), demonstrating that κ rearrangement is not a prerequisite for λ chain assembly. The total number of B cells in the mutant mice is approximately 50% that of wild type (Fig. 3 C,D), representing an ~10-fold increase in the λ^+ B cell population relative to wild type mice. That this increase takes place in the absence of a corresponding increase in the size of the B precursor pool [27, 28] suggests that either the sizes of the individual B cell populations are not subject to feedback regulation, or that in the absence of competition from κ rearrangement, the effective size of the λ precursor pool is increased [for further discussion of the implications of these data for models of light chain isotype regulation see 27–29].

Despite the drastic restriction of the light chain V gene repertoire in κ deficient mice to three V_λ regions, one of which ($V_\lambda X$) is rarely used, these mice have shown themselves capable of compensating for κ deficiency in responses elicited by T independent and T dependent antigens [30]. In addition, the mutant mice appear capable of generating a fully protective neutralizing antibody response to influenza virus in the absence of κ light chains (C. Bona, personal communication) and of eliminating a primary infection with Giardia muris as efficiently as do wild type mice (B. Underdown, personal communication).

HUMAN IMMUNOGLOBULIN MINILOCI

We chose to construct human sequence Ig minilocus transgenes to complement the endogenous Ig-deficiency in the J_HD and JC_KD mutant mice. We did not to attempt to introduce, into the mouse germline, the entire human Ig loci as intact chromosomal fragments because these loci are individually too large to be easily isolated as clones. At 1.3 and ~1.9 Mb respectively [31–33], the sizes of the heavy and κ light chain loci approach the upper size limit of YAC clones. In addition, the Ig loci may contain sequences that are unstable in yeast or bacterial cloning systems. For example, a sequence of ~30 kb located in the heavy chain constant region between δ and γ3 has not yet been cloned, and is coincident with a site of unusually high meiotic recombination [34]. It is possible that such recombinational hotspots will prove to be unclonable. Therefore, as an alternative to isolating the Ig loci as intact clones, we have constructed artificial transgenes—miniloci—that represent edited versions of the natural immunoglobulin loci. In addition to the advantages that small size and low sequence complexity confers on clonability and ease of trangenesis, the modular structure of these minilocus transgenes makes it relatively straightforward to make sequence substitutions within transgenes. For example, it is relatively easy to add or eliminate particular variable or constant genes.

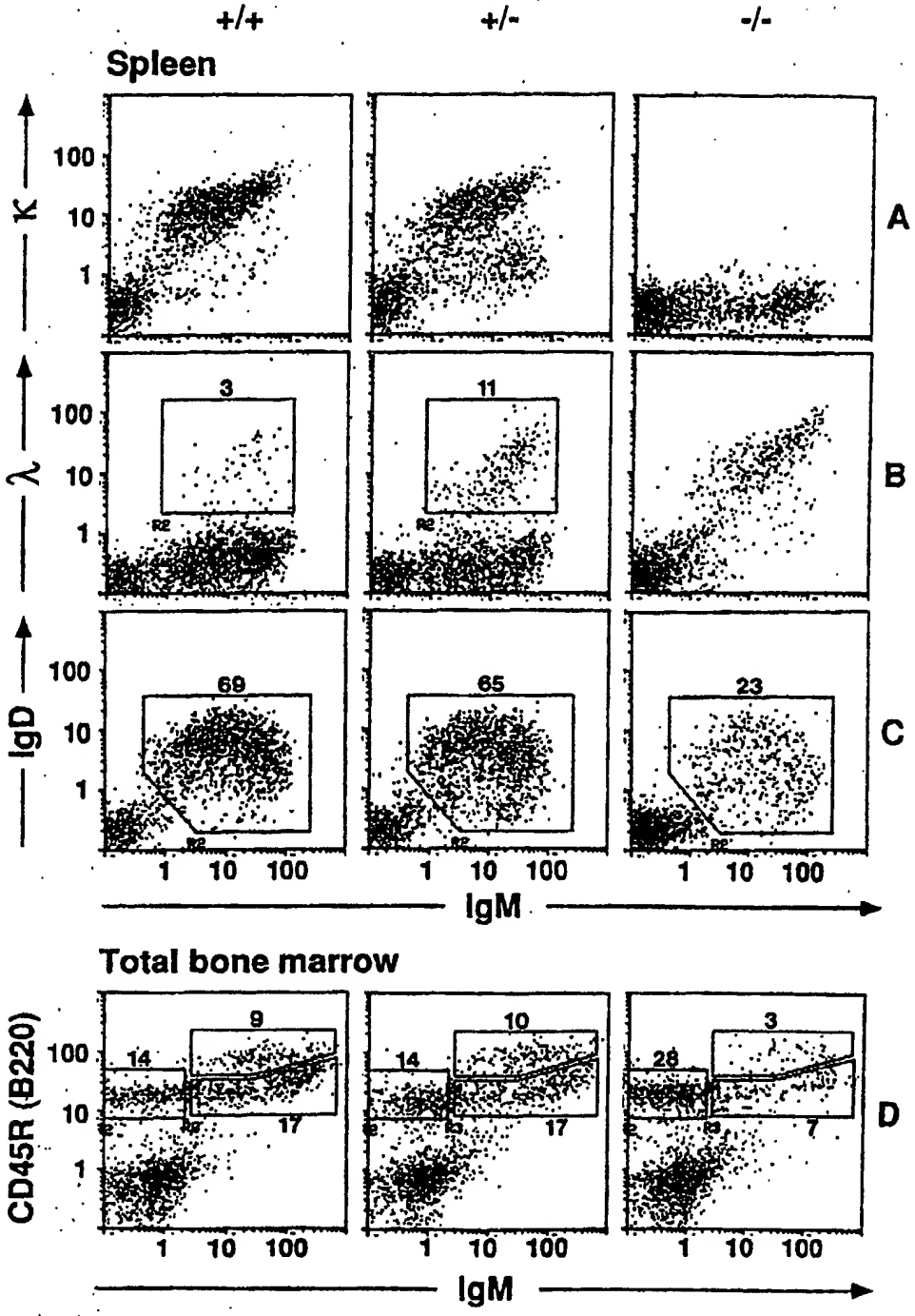


FIGURE 3 Flow cytometric analysis of cells from spleen and bone marrow of homozygous $JC_{\kappa}D$ mutants (−/−), heterozygous mutants (+/−), and wild type (+/+) littermates. At least four mice were analyzed from each genotype. Spleen cells (A, B, and C) were stained with the followihg combinations: fluorescein-anti-κ, PE-anti-λ, and biotin-anti-IgM; or fluorescein-anti-IgD, PE-anti-λ, and biotin-anti-IgM. Bone marrow cells (D) were stained with fluorescein-anti-IgM, and PE-anti-CD45R (B220). Each dot in the two dimensional plots represents an individual cell; the numbers indicate the percentage of cells staining for a particular phenotype in each boxed region. The IgM^+, κ^{dull} stained cell population in heterozygous spleen (panel A) are λ^+ B cells, as suggested by κ versus λ two-dimensional plot (data not shown). These λ^+ B cells are stained by anti-κ antibody probably because they have absorbed serum Ig(κ) molecules through Fc receptors *in vivo*. Note that the λ^+ B cells in κ-deficient mice, which do not make any Ig(κ) molecules, do not stain for κ.

Plasmid Vectors for Minilocus Transgene Construction

Despite the size reductions afforded by discarding non-essential sequences, the minilocus transgenes approach the upper limit for conventional plasmid inserts. We therefore designed new cloning vectors and developed a straightforward general scheme for stepwise construction of large transgenes. These vectors, called pGP vectors [13], are pBR322-based plasmids that are maintained at a lower copy number per cell than the pUC vectors. The vectors also include trpA transcription termination signals between the polylinker and the 3′ end of the plasmid, β-lactamase gene. We have found that these medium copy vectors are an improvement over the commonly used pUC derived cloning vectors. The ability of these vectors to maintain large inserts is illustrated by the experiment shown in Fig. 4. We individually ligated the same 43 kb XhoI fragment, comprising the human JH/Cμ region, into the SalI site of three different vectors; two pUC derived vectors, pSP72 and pUC19, and one pGP vector, pGP1f. Transformant colonies were transferred to nitrocellulose and insert containing clones were selected by hybridization with radiolabeled probe. All the pSP72 and pUC19 derived clones deleted the insert; however, 12 of the 18 pGP1f derived clones contained intact inserts.

The polylinkers of the pGP vectors include two specific features for building large transgenes. First, the polylinkers are flanked by restriction sites for the rare-cutting enzyme NotI; thus allowing for isolation of the insert away from vector sequences prior to embryo microinjection. This is important because vector sequences have been shown to interfere with the proper expression of some microinjected transgenes [35]. And second, internally flanking the NotI sites, the polylinkers include unique XhoI and SalI sites at either end. To build a large construct, all of the component DNA fragments are first cloned individually in the same 5′ to 3′ orientation in pGP vectors (Fig. 5). Individual inserts are then combined stepwise by the process of isolating XhoI/SalI fragments from one clone and inserting the isolated fragment into either the 5′ XhoI or 3′ SalI site of another clone. Because XhoI/SalI joints cannot be cleaved with either enzyme, the resulting product maintains unique 5′ XhoI and 3′ SalI sites, and can be used in the next step of the construction. By the iterative application of this simple process, very large and complex plasmids can be built.

Construction of Heavy Chain Minilocus Transgenes

The natural human heavy chain locus includes ~50 functional V gene segments, ~30 D gene segments, 6 J gene segments, and 9 constant region gene segments [31, 32]. Associated with each of these protein coding segments are cis-acting transcriptional regulatory elements and/or recombination signal sequences. In germline chromosomal DNA these elements are spread out over a distance of 1.3 Mb at the telomere of chromosome 14. To design a minimal functional heavy chain locus, it is necessary to address three issues: repertoire diversity; B cell maturation; and, regulation of gene rearrangement and expression. The issue of repertoire diversity is addressed by including a large enough set of individual V, D, and J elements so that the resulting mouse will be able to respond to a broad range of different antigens. The actual minimal set of germline sequences required for a diverse repertoire is unknown, and certainly cannot be answered until a definition of "diverse" is obtained. The second issue, B cell maturation, must be addressed because the transgene encoded heavy chain is a key component of the B cell receptor that is required for development of this cell lineage; and because isotype switching of the heavy chain is a marker for those differentiated cells involved in the secondary response to antigen. It therefore may be necessary to include the complete, μ gene segment, as IgM is presumably obligatory for proper B cell

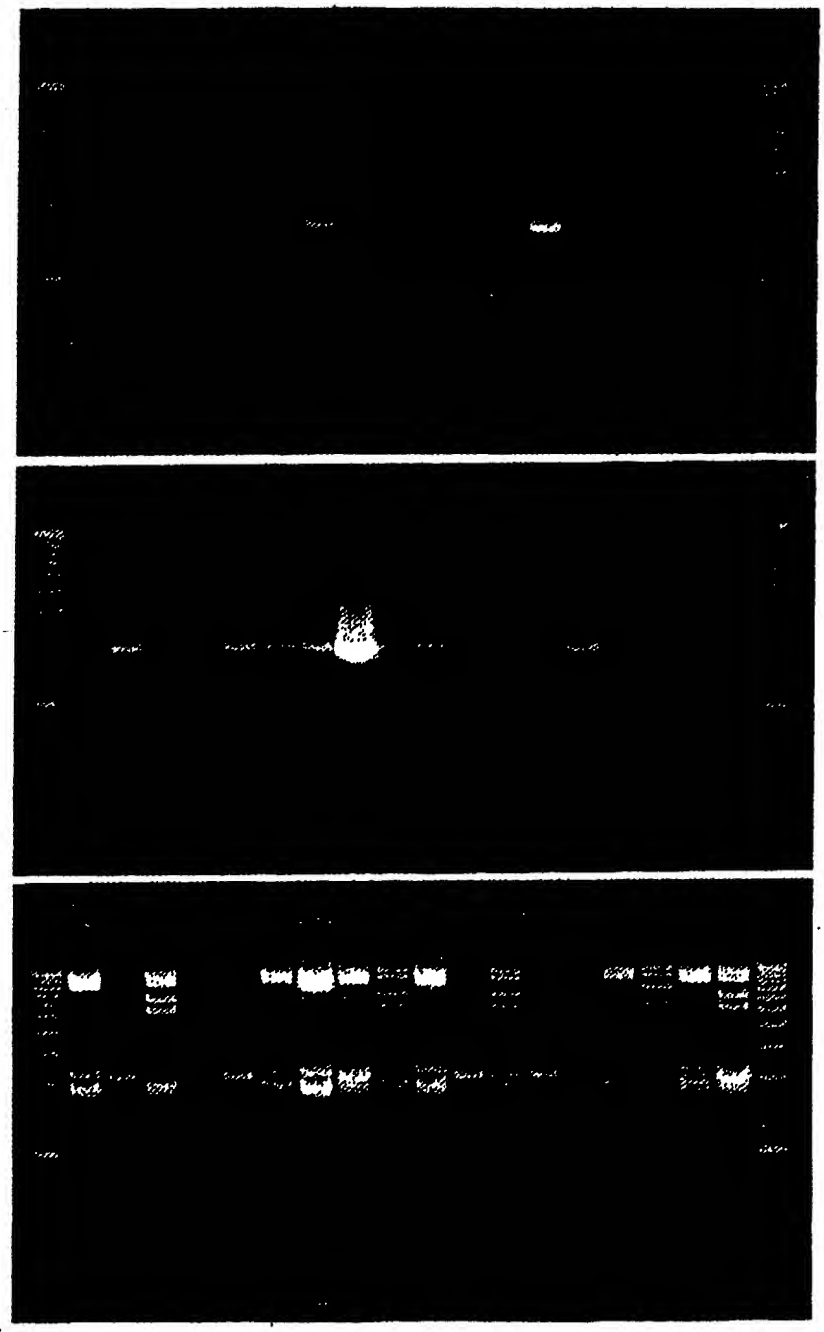
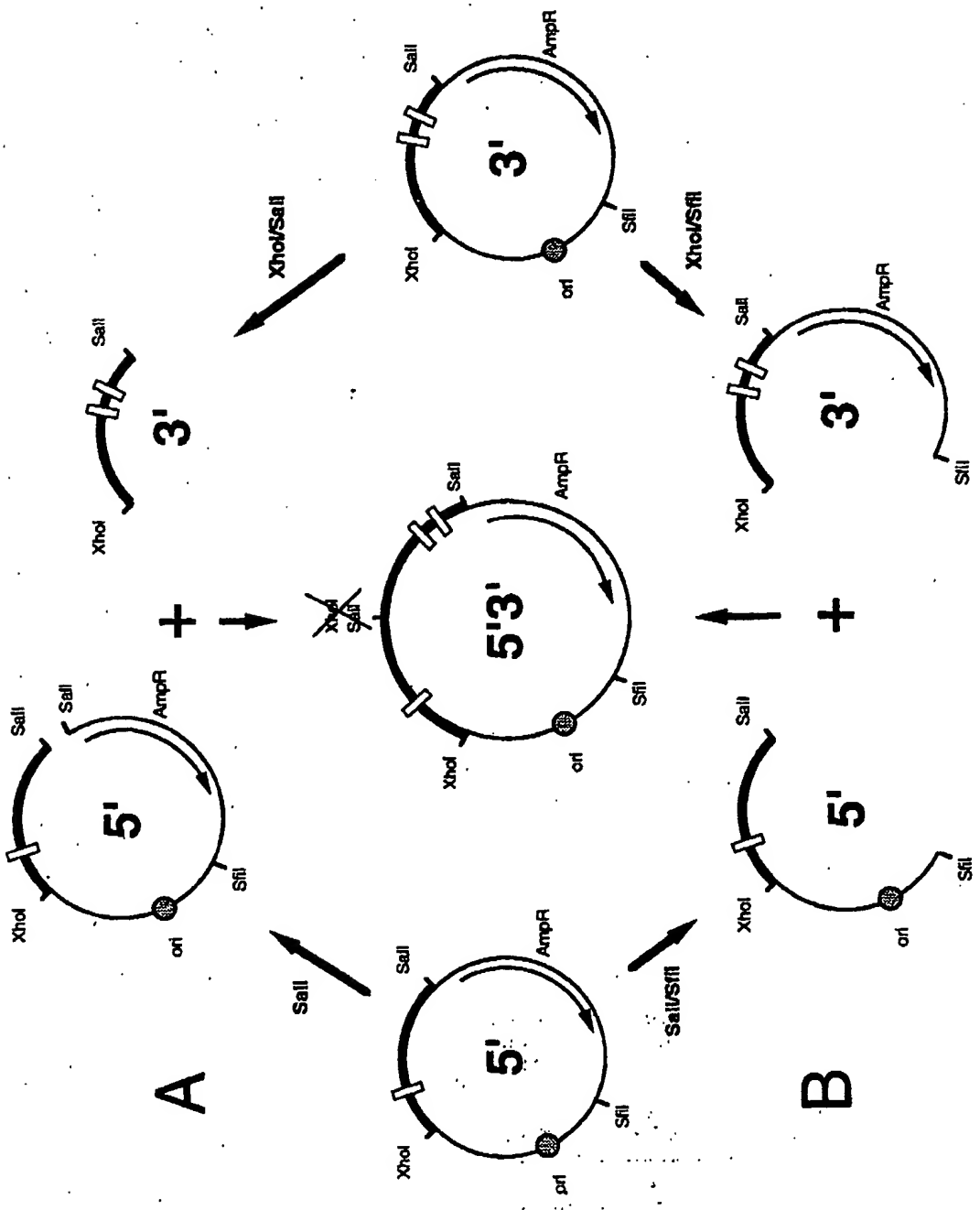


FIGURE 4 Large inserts are more stable in pGP plasmids than in pUC derived plasmids. A 43 kb XhoI fragment comprising the human JH/Cμ region was ligated into the SalI site of pSP72 (Promega, Madison, WI), pUC19 (BRL, Grand Island, NY), and pGP1f. Transformant colonies were transferred to nitrocellulose and clones containing inserts were selected by hybridization with radiolabeled probe. Positive clones were grown overnight in 3 ml media and DNA was isolated and digested with EcoRI.

development, as well as a downstream constant region gene with an associated switch element, so that hybridomas that are derived from affinity matured B cells can be easily identified. The final issue, regulation of gene rearrangement and expression, involves inclusion of all necessary cis-acting regulatory sequences. Many of these sequences—such as recombination signal sequences, RNA splicing signals, transcription initiation and termination sites—are tightly linked to coding exons, and would be included by default in any construct. However, the locus also contains elements, such as enhancers, that act over large distances and affect multiple transcripts. The first enhancer identified within the human heavy chain locus was the J-μ intronic enhancer, which includes flanking matrix attachment sites [36, 37]. This enhancer confers B cell specific expression on immunoglobulin transgenes [38], and is required for efficient VDJ joining within the endogenous mouse locus [39, 40]. A 3′ enhancer has also been identified downstream of the α gene segment in rats and mice [41–43]. The 3′ enhancer appears to be involved in induction of class switching to the γ2a and γ3 isotypes [44], which are encoded, respectively, by gene segments located 60 and 120 kb upstream. However, based on differences in the structure and the pattern of cytokine induction of mouse and human γ genes [45], it is possible that there is no human counterpart of this enhancer element. A second transcriptional element has been identified immediately downstream of the mouse α gene, 12 kb upstream of the so-called 3′ enhancer [46]. This element was found to be specific for lymphoid cell lines, and it could be important for expression of IgA; however, its function has yet to be determined. Other enhancer sequences may be scattered throughout the heavy chain locus. For example, Kottmann *et al.* [47] have reported a weak enhancer upstream of the DQ52/JH cluster. This enhancer could affect the expression of μ germline transcripts, which have been implicated in VDJ joining [48–50]. However, it is unlikely to be the primary control element, as disruption of the J-μ intronic enhancer shuts down the germline transcript [51]. An enhancer element has also been reported upstream of the mouse γ1 switch region [52]. This element appears to control expression of the γ1 sterile transcript, which has been implicated in cytokine mediated induction of class switching [53–55]. Deletion of this element, together with the first exon of the sterile transcript, prevents class switching to γ1 in gene-targeted mice [56]. Analogous sterile transcripts have been identified for each of the other mouse non-μ switch regions [57–62], and gene targeting experiments have demonstrated that sequences upstream of the γ2b switch are required for induction of this isotype [63]. Furthermore, replacement of the ε switch upstream region with a constitutive promoter leads to ε class switching in the absence of cytokine induction in an otherwise switch-inducible cell line [64]. Similar sterile transcripts have been identified for human non-μ switch isotypes, and inducible enhancer elements have been mapped upstream of the human γ3 and ε gene segments [65, 66]. Additional sequences at, or near, the 3′ end of the switch regions may also be important for high level expression of heavy chain isotypes. It has been reported that DNA fragments comprising either the 3′ portion of the human γ1 switch, or the mouse μ switch, confer high level expression on mouse VDJ/human γ1 chimeric transgenes that lack additional switch sequences [67].

We have included many of the sequences listed above in our heavy chain transgenes. In some cases we have included presumptive human homologs of regulatory sequences identified in other species. Our transgenes include: the human μ intronic enhancer, the DHQ52 upstream region, the presumptive human γ1 sterile transcript enhancer, the complete human γ1 switch region and 3′ flanking sequences, and the rat 3′ heavy chain enhancer. However, the size of this list, together with the fact that new regulatory sequences continue to be identified, underscores the risks involved in the minilocus approach. We


A
B
5'
3'
5'3'
XhoI
SalI
AmpR
ori
SfiI
XhoI/SalI
XhoI/SfiI
SalI
SalI/SfiI

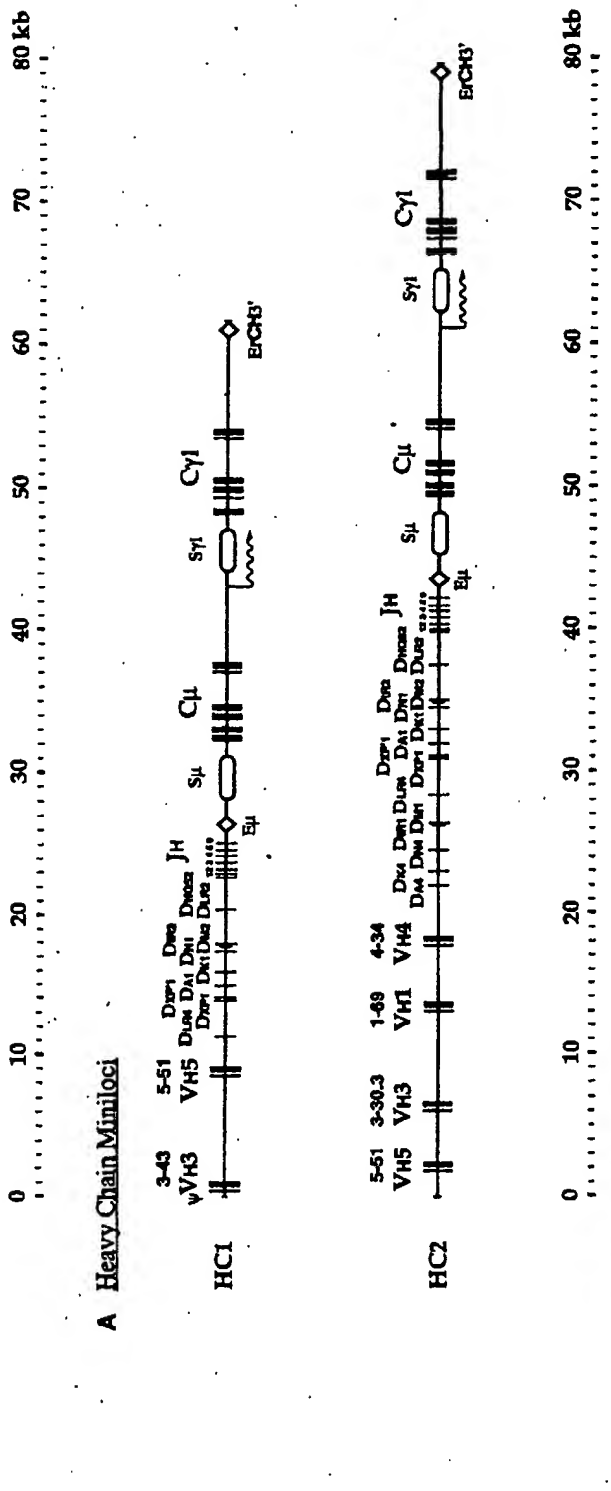
have therefore attempted to include as much flanking sequence as is practical for each of coding elements in our transgenes. We have constructed and microinjected several different heavy chain transgenes. Most of our analysis to date has concentrated on two of these transgenes. These two miniloci, HC1 and HC2, include one and four functional variable (V) segments respectively, 10 and 16 diversity (D) segments respectively, all six joining (JH) segments, and both the μ and γ1 constant region segments [13, 16] (Fig. 6). All of these coding segments are human. The miniloci include human cis-acting regulatory sequences—such as the JH-μ intronic enhancer and the μ and γ1 switch sequences—that are closely linked to the coding segments. They also include an additional enhancer element derived from the 3′ end of the rat IgH locus. Both constructs are propagated as single inserts that can be isolated from pGP plasmid vector sequences by digestion with NotI. The smaller construct, HC1, is a 62 kb insert, while the larger construct, HC2, is an 80 kb insert.

The HC1 transgene contains the single functional V gene segment VH5-51. This V gene segment is one of two members of the VH5 family that have an open reading frame, and it is the only VH5 gene that is known to contribute to the human repertoire [68]. VH5-51 has been reported to be overrepresented in chronic lymphocytic leukemias [68]. There is also a germline transcript associated with this gene in all B and pre-B cells. In addition to this gene, the larger transgene construct, HC2, includes three other functional V genes: VH1-69, VH3-30.3, and VH4-34. VH1-69 is one of 8 members of the VH1 family for which VDJ rearrangements have been reported [32, 69]. This gene was first identified as a component of the human fetal repertoire [70]. VH3υ.3 is a member of the largest VH family, comprising 22 known functional genes [32, 69]. It has also been associated with the fetal repertoire [70]. VH4-34 is a member of the VH4 family, and encodes the 9G4 idiotype [71]. This gene has been found in a number of autoreactive B cell clones, and is associated with cold agglutinin diseases. Although these 4 genes comprise only 8% of the entire library of functional V's, they represent frequently rearranged genes and together they account for approximately one quarter of the reported rearranged gene sequences in the literature [72].

Construction of κ Light Chain Minilocus Transgenes

Although the natural κ light chain locus is larger than the heavy chain locus, the structure is somewhat less complex. In contrast to heavy chain genes, light chain variable regions are assembled from two, rather than three separate elements, and there is no class switching of

FIGURE 5 A plasmid vector for assembling large transgenes. Shown at the far left and right of the figure are DNA fragments from the locus of interest cloned individually in the same 5′ to 3′ orientation in pGP vectors. Insert NotI, XhoI and SalI sites are destroyed by oligonucleotide mutagenesis or if possible by partial digestion, polymerase fill-in, and blunt end ligation, leaving only the polylinker derived XhoI and SalI sites at the 5′ and 3′ ends of each insert. In strategy A, the XhoI/SalI insert from one clone, in this case the 3′ genomic clone, is isolated and ligated into the SalI site of the 5′ genomic clone. The resulting plasmid, shown in the center of the figure, contains the two ligated inserts and maintains unique flanking XhoI and SalI sites for subsequent cloning steps. A variation of this scheme, using the vector pGP2b, which includes an SfiI site between the ampicillin resistance gene and the plasmid origin of replication, is shown in strategy B. By cutting with SfiI and XhoI or SalI, inserts can be isolated together with either the drug resistance sequence or the origin of replication. One SfiI/XhoI fragment is ligated to one SfiI/SalI fragment in each step of the synthesis. There are three advantages to this scheme: (i) background transformants are reduced because sequences from both fragments are required for plasmid replication in the presence of ampicillin; (ii) the ligation can only occur in a single 5′ to 3′ orientation; and (iii) the SfiI ends are not self-compatible, and are not compatible with SalI or XhoI, thus reducing the level of nonproductive ligation.


A Heavy Chain Miniloci
0
10
20
30
40
50
60
70
80 kb
HC1
3-43
ψVH3
5-51
VH5
DQ52
DXP1
DM1
DM2
DM3
DXP1
DXP2
DM4
DM5
DM6
DXP2
JH
Eμ
Sμ
Cμ
Sγ1
Cγ1
EcCH3'
HC2
5-51
VH5
3-30.3
VH3
1-69
VH1
4-34
VH4
Eμ
Sμ
Cμ
Sγ1
Cγ1
EcCH3'
B Light Chain Miniloci
KC1
A27
Vκ3
Jκ
Cκ
Eκ
KCo4
L20
Vκ3
A27
Vκ3
L15
Vκ1
L8
Vκ3
Vκ3
Jκ
Cκ
Eκ
Eκ3'

light chain genes during B cell development. The κ locus consists of ~30 functional V gene segments divided between two clusters separated by 800 kb, 5 J gene segments, and a single constant region gene segment [73]. The entire locus spans ~1.9 Mb and is located on chromosome 2 [33]. Like the rodent heavy chain locus, the κ locus contains a J-C intronic enhancer as well as a 3′ enhancer [74]. Both of these enhancers have been identified in the human κ locus [75], and have been included in our light chain transgene, KCo4 [17] (Fig. 6).

The KCo4 κ-light chain transgene is derived from the cointegration of two individually cloned DNA fragments at a single site in the mouse genome. The fragments together comprise 4 functional Vκ segments, 5 J segments, the Cκ exon, and both the intronic and downstream enhancer elements. All of the sequences are of human origin. Because the two fragments share a common 3 kb sequence, they can integrate into the mouse genome as a contiguous 43 kb transgene following homologous recombination between the overlapping sequences. An analysis of light chain transcripts from two independent lines reveals all four of the transgene encoded V segments are incorporated into the light chain repertoire, and that there is no expression bias in favor of the J proximal V segment, which is the only V that is physically linked to Cκ prior to microinjection (Fig. 7). The expression of V segments from both fragments thus demonstrates the success of the coinjection strategy.

Complementation of Mouse IgH and Igk Mutations by Human Transgenes

To generate mice expressing predominantly human sequence antibodies, we bred animals carrying the human heavy and light chain miniloci together with the J_HD and JC_KD mutant strains. We find that the human heavy chain minilocus transgenes HC1 and HC2 rescue B cell development in mice carrying the J_HD heavy chain mutation [16, 17]. We also find that the human KCo4 light chain transgene is functional, and competes successfully with the intact endogenous λ light chain locus in JC_KD mice homozygous for κ light chain disruption. In KCo4/HC2 transgenic mice that are homozygous for both the J_HD and JC_KD mutations (double-transgenic/double deletion mice) nearly 95% of the $B220^{hi}$ cells express completely human cell surface IgMκ [17]. We find human IgM expressing cells in the spleen, lymph-nodes, peritoneum, and bone marrow of the double-transgenic/double-deletion mice (Fig. 8, and reference 17). Although the peritoneal cavity contains the normal number of B cells, the absolute number of transgenic B cells in the bone marrow and

FIGURE 6 Human sequence immunoglobulin minilocus transgenes. The constructs are described in detail elsewhere [13, 16, 17]. (A) The HC1 heavy chain minilocus contains a single functional variable gene segment, 10 functional D segments, all 6 JH segments, the μ and γ1 constant region segments, as well as the μ and γ1 switch regions. It also contains the human heavy chain J-μ intronic enhancer and the rat heavy chain 3′ enhancer. The HC2 plasmid insert is similar to the HC1 insert; however, it includes 3 additional variable gene segments, and 5 additional D segments, as well as a further 0.7 kb of human γ1 upstream sequence not included in HC1. All of the sequences in HC1 and HC2 are human with the exception of the rat 3′ enhancer. The entire 63 kb HC1 and 80 kb HC2 transgenes are isolated from vector sequences by digestion with Not I. The start site of the human γ1 pre-switch sterile transcript is indicated by the wavy arrow below HC1. (B) The light chain minilocus, KC1 contains a single Vκ segment, all 5 human Jκ segments, the human κ constant region segment, and the intronic enhancer. The light chain minilocus, KCo4, is created by co-injection of two overlapping DNA fragments, each of which is propagated within a different plasmid. One of the DNA fragments includes 4 different functional κ variable segments. The second fragment includes one of these same variable sequences together with all 5 human Jκ segments and the human κ constant region segment. This fragment also includes both the κ intronic enhancer and the 3′ κ enhancer. All of the sequences in these two inserts are of human origin. V, variable segment; D, diversity segment; J, joining segment, C, constant region gene; S, switch region; E, enhancer.

	V_κL20	J_κ2
	CAGCAGCGTAGCAACTGGCATCC	GTACACTTTTGGCCAGGGG
8490-50	----------	----------
	V_κA27	J_κ1
	CAGCAGTATGGTAGCTCACCTCC	GTGGACGTTCGGCCAAGGG
8867-56	----------A---CA-	----------
8867-58	----------CA-	----------
8867-59	----------CA-	----------
8867-60	----------CA-	----------
8867-62	----------CA-	----------
8867-64	----------GGA-	----------
8867-65	----------CA-	----------
8867-66	----------A-	----------
8867-67	----------CA	----------
8867-70	----------CA-	----------
		J_κ2
		GTACACTTTTGGCCAGGGG
8867-63	----------	----------
		J_κ4
		GCTCACTTTCGGCGGAGGG
8490-44	----------	----------
8490-49	----------	----------
		J_κ5
		GATCACCTTCGGCCAAGGG
8490-52	----------	----------
	V_κL15	J_κ1
	CAACAGTATAATAGTTACCCTCC	GTGGACGTTCGGCCAAGGG
8490-39	----------	----------
8490-41	----------	----------
8490-47	----------	----------
8867-57	----------	----------
8867-71	----------	----------
		J_κ4
		GCTCACTTTCGGCGGAGGG
8490-37	----------	----------
8490-38	----------TC	----------
8490-45	----------	----------
8867-72	----------	----------
		J_κ5
		GATCACCTTCGGCCAAGGG
8490-40	----------	----------
8867-55	----------	----------
	V_κL6	J_κ1
	CAGCAGCGTAGCAACTGGCCTCC	GTGGACGTTCGGCCAAGGG
8490-46	----------	----------
8490-53	----------	----------
8867-68	----------A	----------
8867-69	----------	----------
		J_κ2
		GTACACTTTTGGCCAGGGG
8490-43	----------	----------
		J_κ3
		ATTCACTTTCGGCCCTGGG
8490-42	----------	----------
		J_κ4
		GCTCACTTTCGGCGGAGGG
8490-48	----------	----------
8490-54	----------	----------
8867-61	----------A-A-	----------
		J_κ5
		GATCACCTTCGGCCAAGGG
8490-51	----------	----------

FIGURE 7 Nucleotide sequence of VJ junctions formed by rearrangement of human κ chain transgene KCo4. The partial sequence of Vκ regions from light chain cDNA clones isolated from two independent KCo4 transgenic lines (mouse #8490, 3 mo., male, KCo4 line 4437; mouse #8867, 2.5 mo., female, KCo4 line 4436). All four transgene encoded V gene segments and all five J gene segments are represented in this set. Experimental details are published elsewhere [17].

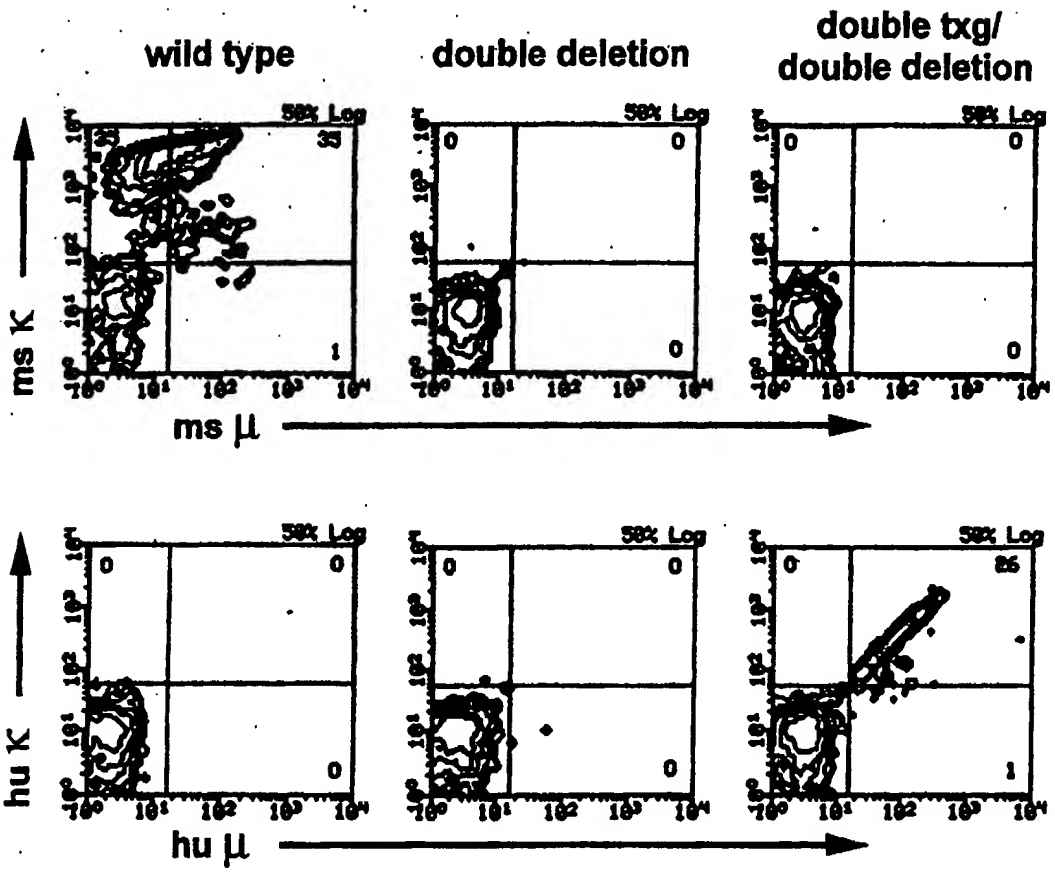


FIGURE 8 Minilocus transgenes rescue the splenic B cell compartment in double deletion mice. Flow cytometric analysis of fluorescent stained cells isolated from spleens of 3 mice with different genotypes. For each of the 2-color panels, the relative number of cells in each of the displayed quadrants is given as percent of a 3-parameter, live-lymphocyte, gate based on propidium iodide staining and light scatter. Left column: control mouse (#9944, 6-week-old female JH +/−, JCκ +/−). Middle column: double deletion mutant $J_HD/JC\kappa D$ animal (#9878, 6-week-old female JH −/−, JCκ −/−, KCo4 line 4437 +). Right column: double transgenic/double deletion mutant (#9879, 6-week-old female JH −/−, JCκ −/−, HC2 line 2550 +, KCo4 line 4437 +). Top row: expression of mouse μ heavy chain (x-axis) and mouse κ light chain (y-axis). Bottom row: expression of human μ heavy chain (x-axis) and human κ light chain (y-axis).

spleen is typically 10–50% of normal. The observed reduction could be the result of a retardation in transgene dependent B cell development. This would have less impact on a population of self renewing B cells, such as that found in the peritoneum, than on the bone marrow and spleen B cell populations, which require a constant supply of newly formed cells. The double-transgenic/double-deletion mice also express fully human antibodies in the serum. We find significant levels of human μ, γ1, and κ in these animals [17].

To test the ability of the transgenic B cells to participate in an immune response with the production of antigen specific human antibodies, we immunized the mice with human protein antigens, and monitored serum levels of antigen specific immunoglobulins. The initial antibody response against injected human IgE is a human IgMκ α-IgE response, followed three weeks later by the emergence of antigen specific human IgGκ [17]. This lag prior to the appearance of IgG antibodies is consistent with an association between class switching and a secondary response to antigen. Repeated exposure to human antigens leads

to relatively high serum titers of specific human IgG. In serum from a transgenic mouse immunized with recombinant human CD4, human IgG reactivity to antigen is detectable at a dilution of one part in ten thousand [17].

Isolation of Human Sequence Monoclonal Antibodies from Double-Transgenic/ Double-Deletion Mice

Because the human immune system reacts to rodent monoclonal antibodies as foreign proteins [76], it is desirable to obtain human monoclonal antibodies for *in vivo* therapeutic use. However, authentic human antibodies are difficult to obtain. In addition, many potential therapeutic targets are human antigens [77, 78], and the phenomenon of immunological tolerance further limits access to authentic human antibodies that are reactive to human antigens. Most of these few available auto-reactive human antibodies are therapeutically uninteresting: they are largely low affinity IgM molecules that bind to broadly distributed intracellular antigens [79–81]. However, by transferring the human immunoglobulin genes into the mouse germline, we have isolated the resulting transgenic B cells from the rest of the human immune system; and in the process, we have gained access to those human antibodies specific for human antigens that are normally suppressed by tolerance. The two previously cited examples, antibody responses to human IgE and human CD4 proteins, illustrate the particular utility of the transgenic mouse. Unlike humans, the transgenic mouse is not tolerant to human protein antigens, and responds to exposure to these antigens with the production of specifically reactive human IgGκ. We isolated hybridoma cell lines from fusions of mouse myeloma cells with splenocytes from mice that responded to human CD4 immunization. We have identified and cloned both human IgMκ and human IgGκ secreting cell lines that bind to recombinant human CD4 and do not cross-react with a panel of other glycoprotein antigens [17].

Class Switching and Somatic Mutation

In addition to their practical utility for generating potential human therapeutics, we are using the transgenic mice to study the normal processes of immunoglobulin gene expression. Class switch recombination of introduced heavy chain transgenes has been previously reported [82, 83]. However, because the trans-switch recombination events described in those experiments involved transgene encoded μ and endogenous γ switch sequences, they do not completely define the cis-acting sequences required for switching. While we also observe trans-switching in our system, our introduced heavy chain minilocus transgenes include both μ and γ1 switch regions, and are thus capable of undergoing intra-transgene class switch recombination. Furthermore, transgene-autonomous class switching in the transgenic mice appears to be taking place in the same population of B cells that are involved in the secondary response to antigen, suggesting that the human transgene class switch could be regulated in mouse cells. This is demonstrated by the observation that somatic mutation of the heavy chain transgenes is largely confined to the products of class switching (Table I). We determined the V region nucleotide sequence of 47 heavy chain cDNA clones from a single HC1 transgenic animal; 24 of the clones were μ and 23 were γ. The density of non-germline encoded nucleotides in the μ population is low (0.1%), and is indistinguishable from a poisson distribution (Fig. 9, top panel). However, the overall density of nongermline encoded nucleotides within the γ population is an order of magnitude higher (1.2%), and does not fit a poisson distribution (Fig. 9, bottom panel). It appears

TABLE I

The frequency of non-germline encoded nucleotides in cDNA sequences of VH genes from transgenic mice

Mouse No.	Age (months)	Transgene	Isotype	No. sequences	Nucleotides sequenced	Observed nucleotide changes	% nucleotide substitution
2599	2	HC1	γ	13	3,197	2	0.1
3204	7	HC1	γ	13	3,192	52	1.6
2357	3	HC1	μ	24	5,904	8	0.1
2357	3	HC1	γ	23	5,658	66	1.2
5250	2	HC2	γ	23	5,644	106	1.9

that the γ clones are derived from at least two distinct populations: a minority (approximately 20%) of the clones contain few non-germline encoded nucleotides, while the majority (approximately 80%) are derived from cells that have undergone multiple rounds of somatic mutation. The distribution of these somatic mutations across the transgene V regions is not uniform. The highest density of non-germline encoded nucleotides occurs within the CDR1 region; and there are specific "hot spots" that accumulate the most mutations. These "hot spots" correspond well to codons that are frequently mutated in normal human cells (Fig. 10). The four codons that are found to be most frequently mutated in human B cells are also the four most frequently altered codons in the transgenic B cells (Fig. 11). The pattern of somatic mutation thus appears to be an intrinsic property of the V region sequence, and is unlikely to be dependent on a template provided by other V, or V-like, sequences elsewhere in the genome. An additional property of the observed somatic mutations, further suggesting that the transgenes are participating in a secondary response, is the replacement-to-silent (R/S) ratios for framework and CDR regions. There appears to be a suppression of replacement changes within the transgene encoded V gene framework regions, and a selection for replacement changes in CDR regions (Table II). For a set of 68 γ sequences encoding VH5-51 or VH3-30.3, the observed R/S ratios for framework and CDR regions are 2.7 and 6.4 respectively. The expected random R/S ratios for these same sequences are 4.0 and 4.0.

In summary, three different qualities of the observed non-germline encoded nucleotides in the transgene V sequences indicate that the transgenic cells are participating in a secondary response, and that class switching of the human sequences is part of this response. These three qualities are: (i) the confinement of somatic mutation to γ sequences; (ii) the similarity of the pattern of somatic mutations in human and transgenic B cells; and (iii) the distortion of R/S ratios in CDR and framework regions.

In normal mice there is abundant evidence that class switching is regulated by sequences associated with each of the individual non-μ switch regions. Individual antigen-activated B cells enter distinct maturation programs determined by the levels and types of cytokines and other switch-factors they are exposed to, and marked by the isotype of the class switch they undergo. For example, in mouse B cells LPS and interleukin 4 (IL-4) specifically induce switching to IgG1 and IgE, while LPS and interferon-γ specifically induce switching to IgG2a. Prior to switching, these same factors specifically induce sterile transcripts that initiate upstream of each of the switch regions [57–62]. The observed correlation between factors that induce sterile transcripts of specific isotypes, and factors that promote expression of those same isotypes, suggests a mechanistic link between switching and transcription. Although the details of this mechanism are obscure, it is likely that the two processes

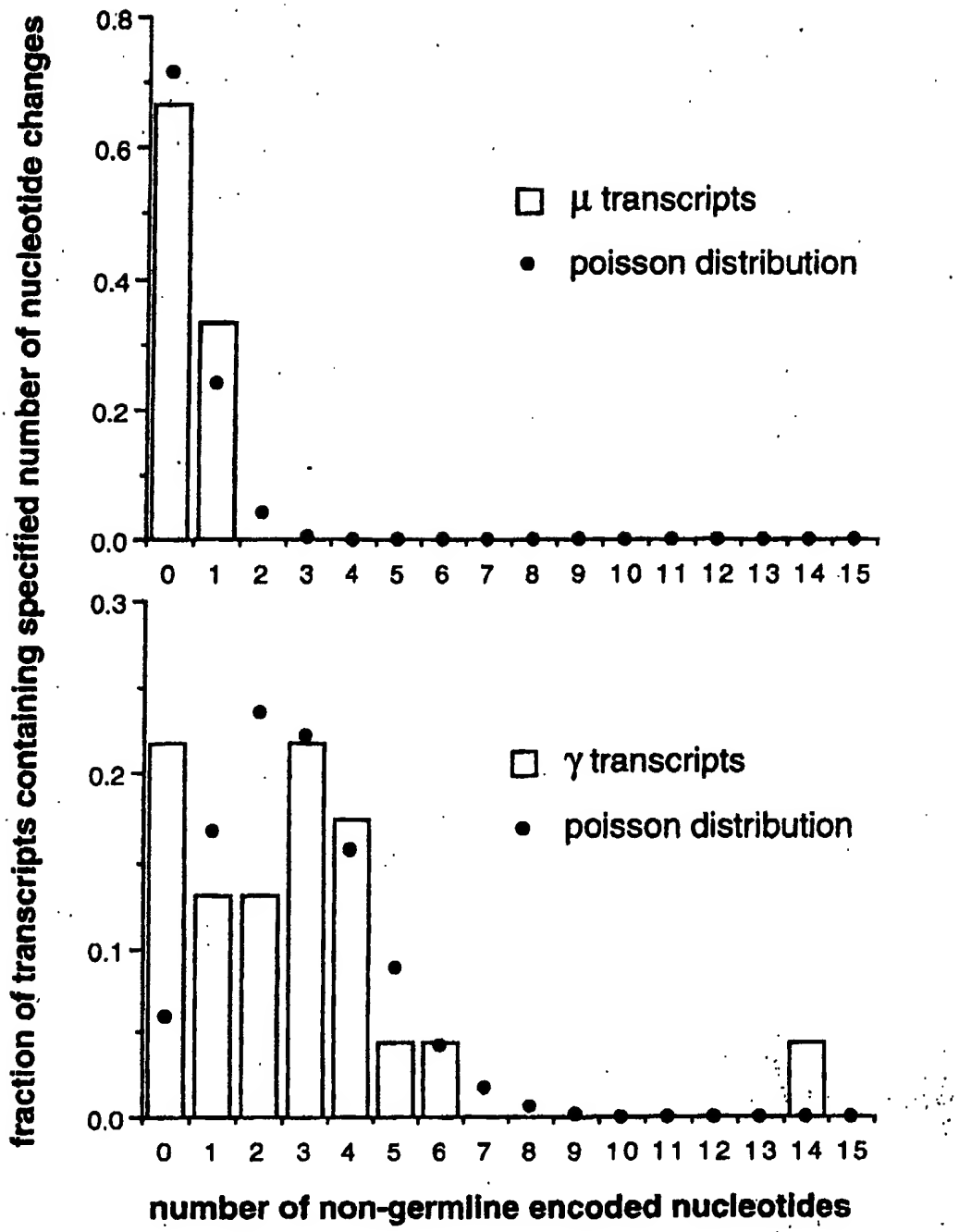


FIGURE 9 Density of observed nucleotide changes per cDNA clone in μ and γ transcripts from a single transgenic mouse. The nucleotide sequence was determined for μ and γ cDNA clones isolated from the same RNA sample taken from the spleen of a single HC1 transgenic mouse [5]. Top panel: data from 24 μ sequences shown as open bars; poisson distribution for this average density shown as filled circles. Bottom panel: data from 23 γ sequences shown as open bars; poisson distribution for this average density shown as filled circles.

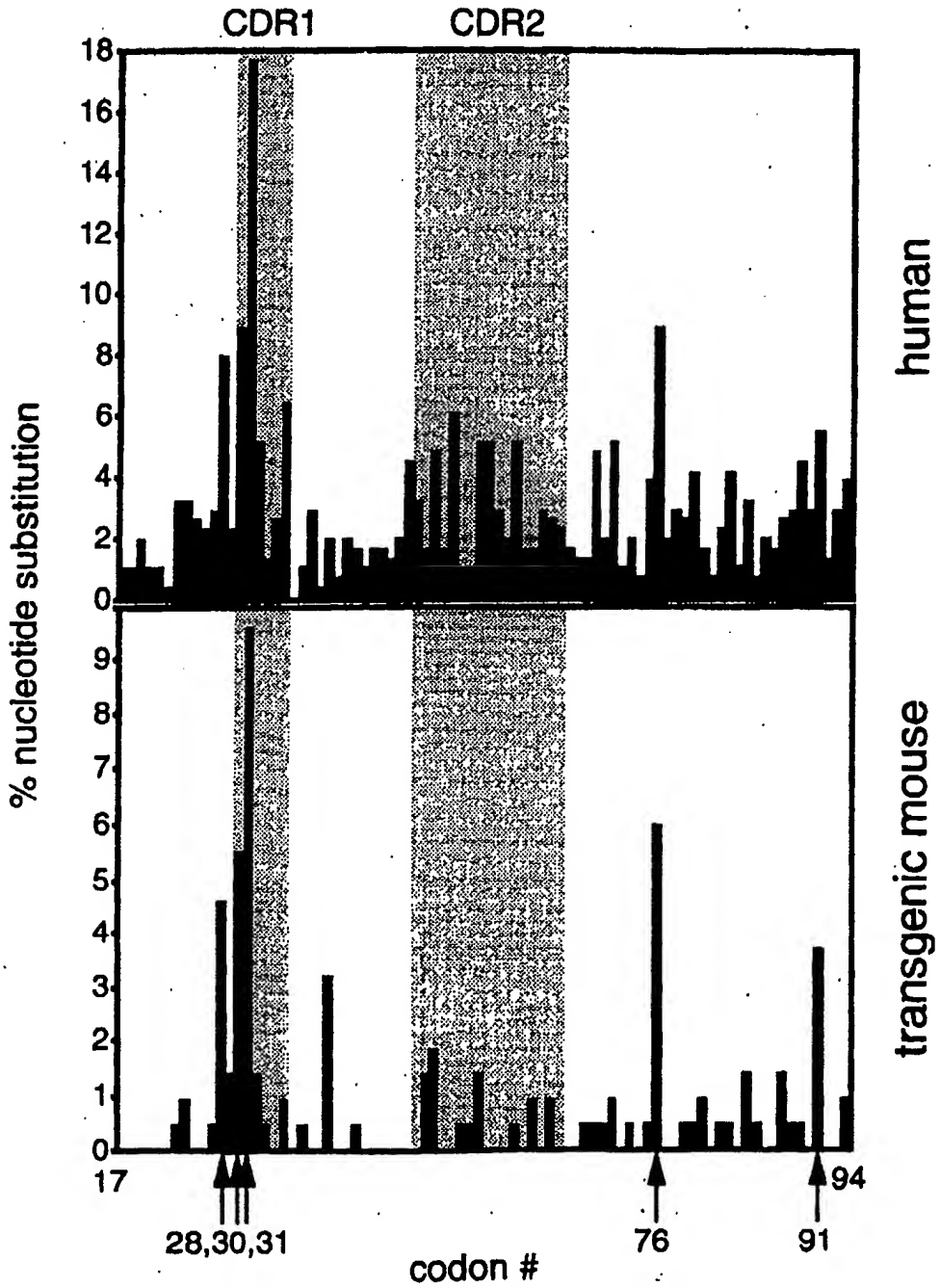


FIGURE 10 Distribution of somatic mutations within VH251 transcripts expressed in humans and transgenic mice. Somatic mutation "hot spots" within the VH251 gene are similar in authentic human B cells and B cells from transgenic mice. Top: distribution of non-germline encoded nucleotides within 106 authentic human VH 251 sequences from the published literature [90–93]. Bottom: distribution for 73 VH251 sequences (49 γ and 24 μ cDNA clones) from HC1 transgenic mice [16].

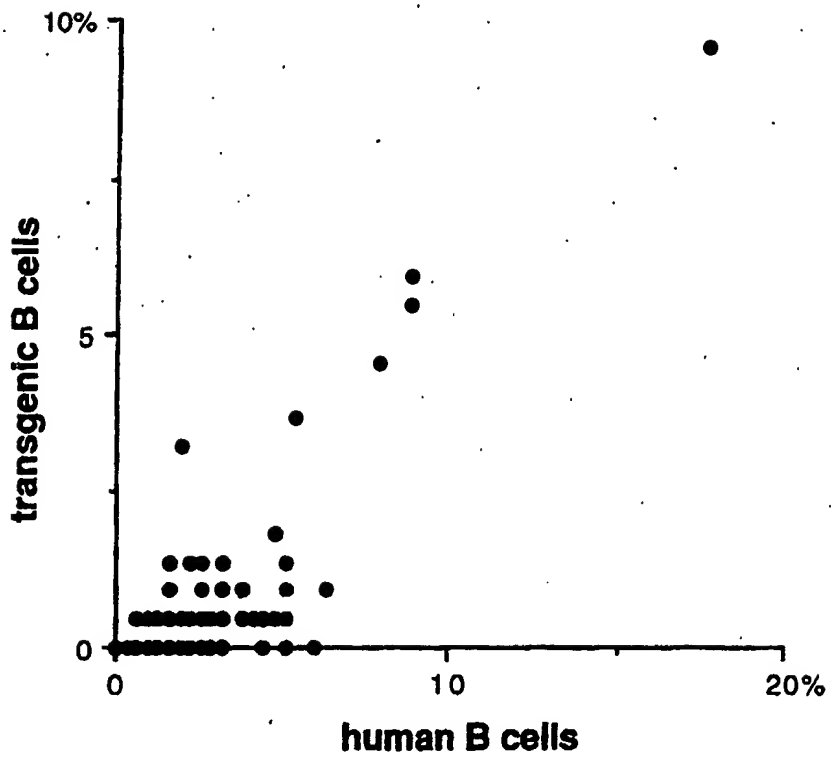


FIGURE 11 Comparison of observed nucleotide changes within individual codons of the cH5-51 gene expressed in human vs. transgenic mouse B cells. The data from Figure 10 (bottom panel) is replotted to show the correlation between nucleotide changes in transgenic mouse and authentic human B cells for individual codons of the VH5-51 gene.

are controlled by the same cis-acting DNA sequences. Sequences that are closely linked to the initiation sites of the sterile transcripts are clearly important; for example, targeted deletion of the first exon of the mouse γ1 or γ2b sterile transcript, together with upstream flanking sequences, eliminates class switching to γ1 and γ2b respectively [84, 85]. These results establish that upstream cis-acting sequences define the functionality of the individual switch regions, and are necessary for class switching. Our observation—that class

TABLE II

The ratio of replacement vs. silent changes in transgene encoded VH transcripts

VH gene segment		Replacement changes	Silent changes	R/S expected	R/S observed‡
VH5-51*	CDR1	33	6	4.4	5.5
	CDR2	12	5	3.5	2.4
	FR	50	22	4.3	2.3
VH3-30.3†	CDR1	26	1	6.7	26.0
	CDR2	31	5	3.8	6.2
	FR	31	7	3.1	4.4

*73 sequences (24 μ, 49 γ)
†19 sequences (all γ)
‡For all 68 γ sequences from both V genes, the R/S ratios are: observed, R/S_{CDR} = 6.4, R/S_{FR} = 2.7; expected, R/S_{CDR} = 4.0, R/S_{FR} = 4.0

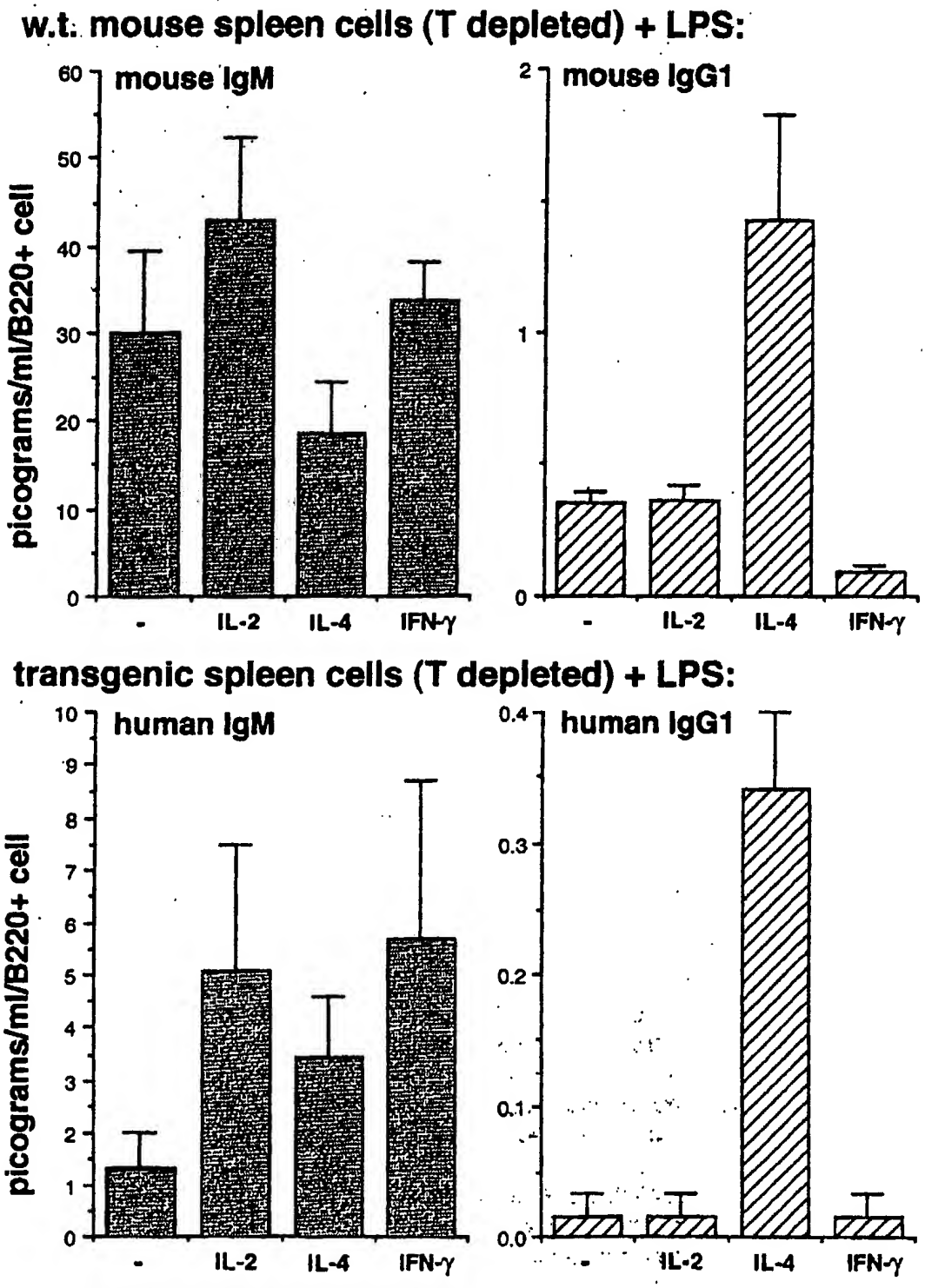


FIGURE 12 Cultured transgenic B cells undergo regulated class switching to the transgene encoded human γ1 isotype. Splenic B cells from wild-type control, and double deletion/double transgenic mice were tested for immunoglobulin secretion in response to the thymus-independent B cell mitogen, LPS, alone and in conjunction with various cytokines. Experimental details are published elsewhere [97].

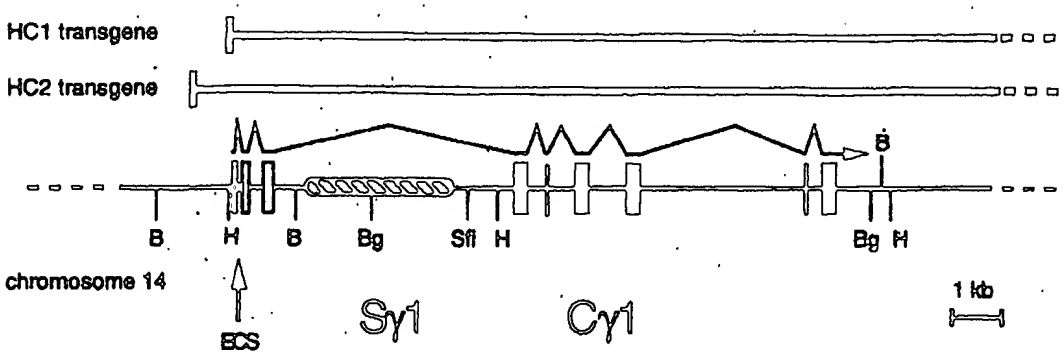


FIGURE 13 Human γ1 isotype switch control region. The germline chromosomal organization of the human γ1 gene and switch region, including exons encoding the pre-switch sterile transcript. The chromosomal sequences included in the HC1 and HC2 transgenes are indicated above the map. Open boxes, sterile transcript exons; filled boxes (Cγ1), γ1 coding exons; shaded ellipse (Sγ1), switch regions; ECS, evolutionarily conserved sequence [87–89]. Restriction sites: B, BamHI; H, HindIII; Bg, BgIII; Sfi, SfiI.

switching within the HC1 transgene is largely confined to cells involved in secondary responses, and does not occur randomly across the entire B cell population—suggests that the minimal sequences contained within the transgene are sufficient. This result is supported by *in vitro* experiments with cultured B cells from the double transgenic/double deletion mice [97]. Transgenic B cells can be specifically induced to secrete human γ1 by LPS and IL-4 under the same conditions that induce mouse γ1 in wild type mouse B cells (Fig. 12). Because the γ sequences included in the HC1 construct begin only 116 nucleotides upstream of the start site of the γ1 sterile transcript (the HC2 transgene includes an additional 0.7 kb of upstream sequence), the switch regulatory region appears to be compact (Fig. 13).

Because of species differences in the expression of heavy chain constant region genes, it is somewhat surprising that a human γ1 switch region is regulated at all in mouse cells *in vivo*. Structural comparisons of mouse and human γ genes, as well as differences in IgG subclass responses, suggest that the individual subclasses evolved independently in the two lineages [45]. While individual mouse IgG isotypes are induced *in vitro* by unique combinations of switch factors, it is not clear if there is differential regulation of the human IgG isotypes. In fact it has been reported that all four of the human γ isotypes can be induced by IL-4; whereas mouse IL-4 specifically induces only mouse γ1 (Table III). A possible explanation for the observation that expression of the human γ1 gene is regulated in transgenic mouse B cells can be obtained from an inspection of the nucleotide sequences upstream of the human and mouse switch regions (Fig. 14). The upstream sequence has been previously reported to include a region of nucleotide sequence that is conserved across all of the mouse and human γ genes [86–88]. This so-called "evolutionary conserved sequence" (ECS) is coincident with a fragment of DNA from the mouse γ1 gene that has been reported in the literature to confer IL-4 inducible transcription on a reporter gene, and is presumed to include the IL-4 inducible enhancer that regulates mouse γ1 switching [89]. The sequence homology matrices presented in Fig. 14 show that, within this conserved region, the mouse γ1 gene is more closely related to the human γ1 gene than it is to the

TABLE III

Cytokine induction of class switching in cultured mouse and human B cells

Mouse B cells:	LPS + IL-4*	LPS + IFN-γ**	LPS + TGF-β***
IgG1	+		
IgG2a		+	
IgG2b			+
IgG3			
IgE	+		
IgA			+
Human B cells:	**low IL-4 + EBV†**	**high IL-4 + EBV†**	**IL-10 + TGF-β + α-CD40††**
IgG1	+		
IgG2	+		
IgG3	+		
IgG4		+	
IgE		+	
IgA			+

References: *98, **99, ***100, †101, ††102

mouse γ2b gene. Thus, in terms of regulation of class switching, the human γ1 gene appears to be the functional homologue of the mouse γ1 gene.

CONCLUSION

The germline configuration human sequence Ig miniloci described here can complement endogenous heavy and kappa light chain loci, rescuing B cell development and function in an otherwise B cell deficient mouse. Human Ig expressing transgenic B cells are capable of responding to antigen in the context of the mouse immune system. Antigen responsivity leads to immunoglobulin heavy chain isotype switching and variable region somatic mutation. We have also demonstrated that conventional hybridoma technology can be used to obtain human sequence MAbs from these mice. Therefore, these animals represent a potential source of human MAbs against human target antigens. Furthermore, because they are not tolerant to human antigens—other than those encoded by the introduced transgenes—the mice are particularly useful for obtaining human monoclonal antibodies directed against human antigens. This is a class of compounds that is potentially useful for *in vivo* therapy.

In addition, these mice are useful for studying, and identifying, those cis-acting sequences that regulate the normal processes of immunoglobulin gene expression, such as class switching and somatic mutation. We have demonstrated transgene autonomous class switch recombination of our heavy chain miniloci both *in vivo* in cells undergoing secondary responses to antigen, and *in vitro* following induction with LPS/IL4. This delineates a functional human γ1 switch regulatory sequence, within the heavy chain minilocus, which is regulated in mouse cells and shares homology with the murine γ1 switch regulatory sequence. We have also observed somatic mutation of the heavy chain transgenes, primarily restricted to those miniloci that have undergone a class switch. The distribution of mutations across the transgene V regions is non-uniform, and is very similar to that

observed in normal human B cells, indicating that the pattern of somatic mutation appears to be an intrinsic property of the V gene.

Acknowledgments

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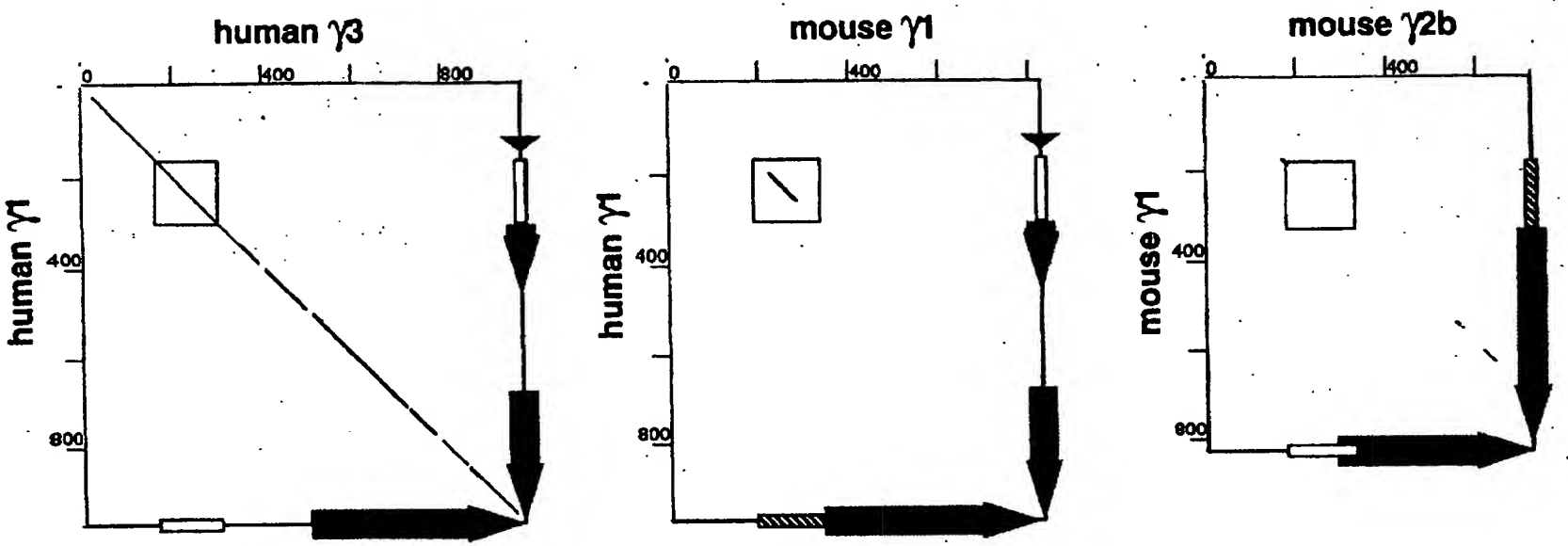

FIGURE 14 Sequence homology between human and mouse γ switch control regions. The nucleotide sequences of the sterile transcript exons and immediate upstream flanking regions of the human γ1 and γ3 genes—and the mouse γ1 and γ2b genes—are compared using a nucleotide identity matrix algorithm. Each point represents a 51 bp window of one sequence with 65% or greater nucleotide identity to the corresponding 51 bp of the other sequence. By this very stringent criteria it can be seen that the individual human γ upstream regions are more closely related to each other than the individual mouse sequences are to each other. And, the human genes include sequences that are homologous to sequences within the mouse genes. The shaded arrows represent sterile transcript exons that have been reported in the literature (human γ1 and γ3 [65], mouse γ1 [94–96], mouse γ2b [58]). The striped box upstream of the mouse γ1 gene represents the 150 bp region shown by Xu and Stavnezer [94] to be sufficient for IL-4 inducible transcription. The corresponding sequence for each of the other γ genes is represented by an open box. The rectangle within each of the matrices indicates the sequence comparison of these corresponding regions. This region includes those upstream sequences that share the greatest degree of sequence identity between the mouse and human genes.

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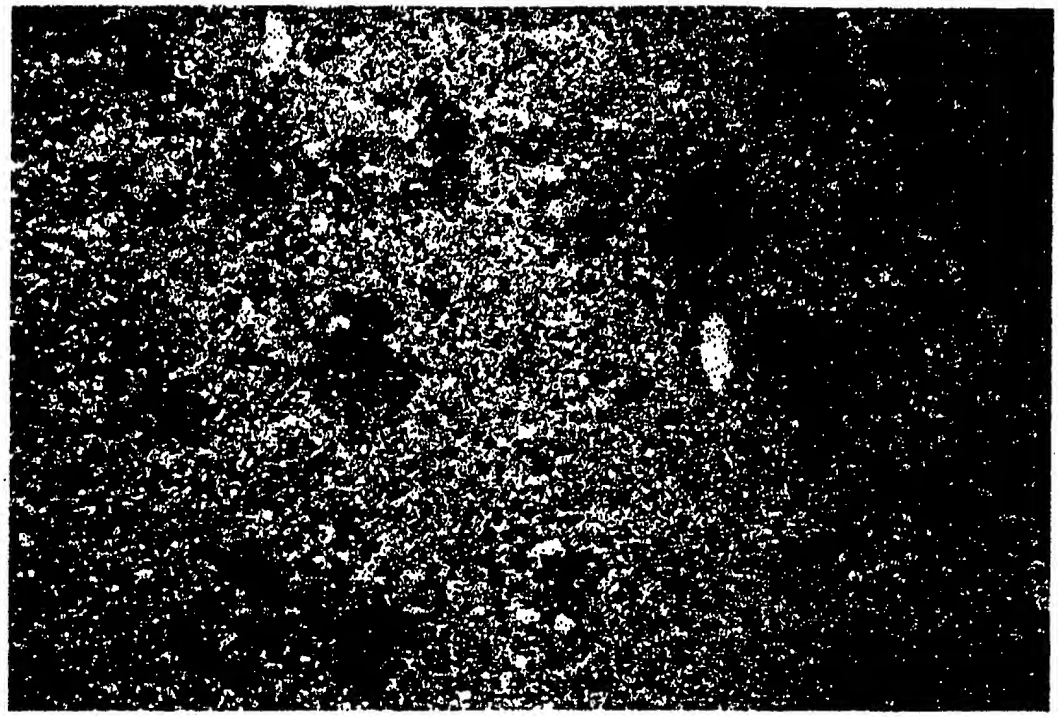

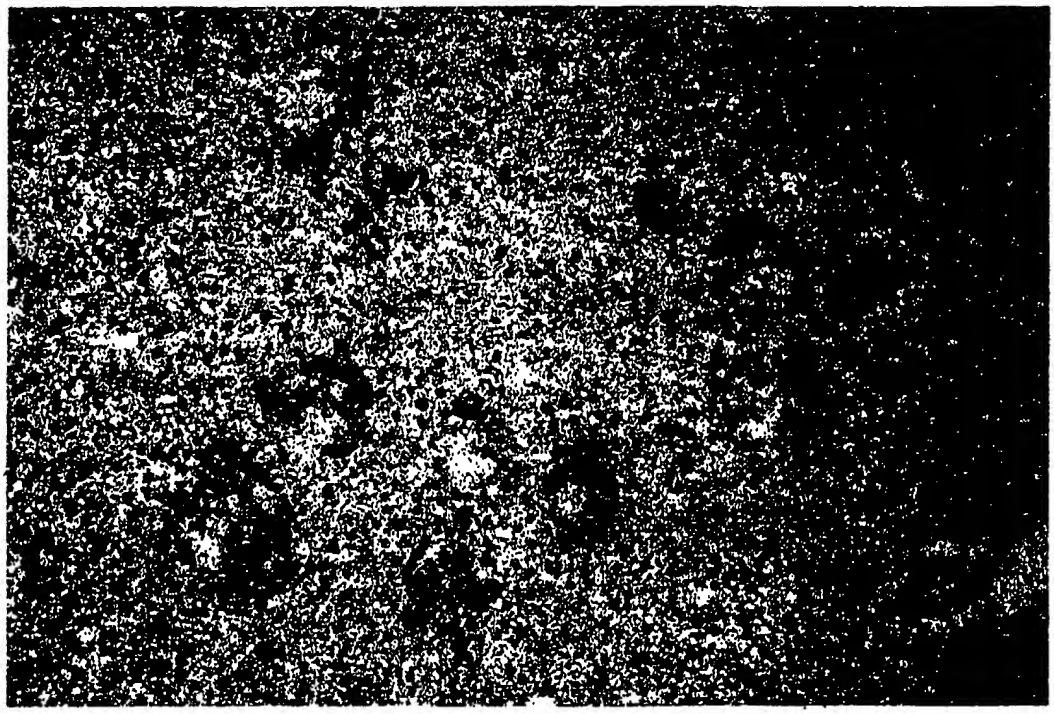

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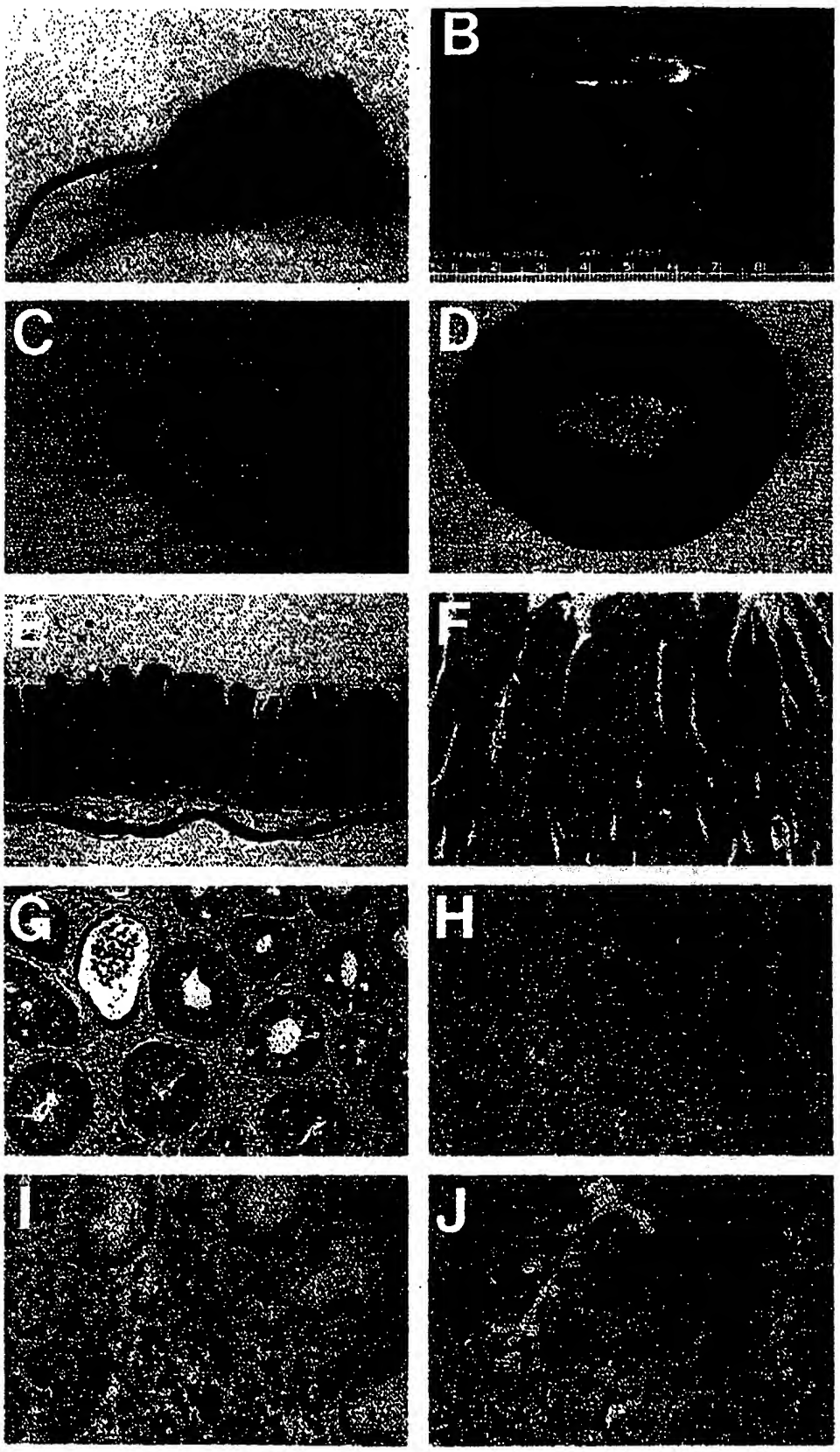

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